Pharmacological strategies for treating misfolded rhodopsin-associated autosomal dominant retinitis pigmentosa

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Mutations that cause protein misfolding are implicated in conditions such as retinitis pigmentosa (RP), Usher Syndrome, and myocilin associated primary open angle glaucoma. The aggregation and continuous degradation of a highly abundant misfolded protein add proteolytic load of the affected cells. The subtle balance of cellular homeostasis, once disrupted by an overwhelmed proteolytic system, will lead to cell death and tissue degeneration. This perspective uses *RHODOPSIN* (*RHO*)-associated RP to review pharmacologic strategies for modifying protein misfolding-associated abnormalities with the goal of bringing insights to the treatment of other proteinopathies.

***RHO* mutations and RP:** RP is the most common inherited retinal degeneration, affecting 1/4000 and, in total, over 1,000,000 people worldwide (Hartong et al., 2006). Genetic mutations lead to abnormalities of rods or retinal pigment epithelia that manifest as nyctalopia and progressive visual field defects beginning peripherally. As the disease advances, central vision is initially maintained; however, eventual secondary loss of cone cells in the fovea can cause legal blindness in some end-stage RP patients. Onset typically occurs in young adulthood and the progression of the disease varies from years to decades. Acquired vision loss severely affects quality of life, and visual disability can additionally have profound psychosocial morbidity. These characteristics highlight the urgent need for safe and efficacious treatment of this currently untreatable disease.

Mutations in the *RHODOPSIN* (*RHO*) gene account for 25–30% of all autosomal dominant RP (adRP) cases. The first identified RP mutation is the *RHO* P23H mutation, which happens to be the most common adRP mutation in the USA. Among the 150+ *RHO* mutations that cause RP, only four mutations are associated with autosomal recessive RP – the remainder cause adRP mainly via dominant negative effects (https://sph.uth.edu/retnet/). The class II *RHO* mutations contain over 50 single mutations that destabilize the native structure of rhodopsin and lead to its misfolding. These mutations are mostly localized on the extracellular loop and plug motifs, as well as within the transmembrane domains.

As a photopigment, rhodopsin is the most abundant protein located in the outer segments (OS) of rods, and it enables the characteristic high photosensitivity (Palczewski, 2006). Binding with an 11-*cis*-retinal chromophore allows photoactivation of rhodopsin and initiation of visual phototransduction. Biosynthesis of rhodopsin reaches about 6 million molecules per day per rod, lasting for the cell's entire life span. The biosynthesis of rhodopsin requires highly coordinated regulation of the *RHO* gene expression, protein translation and folding, glycosylation, palmitoylation, and transport to the OS. Dysregulation of any of these steps can lead to rod stress and death. As a class A G-protein coupled receptor and a membrane protein that is not as stable as soluble proteins, the folding of a nascent apoprotein rod opsin is supported and monitored by molecular chaperones in the endoplasmic reticulum (ER) (Athanasiou et al., 2018). Misfolded rhodopsin activates the ER associated protein degradation (ERAD) pathway, leading to its degradation by the ubiquitin-proteasome system or the lysosome system (**Figure 1A**). Mutations that cause rhodopsin misfolding continuously activate the ERAD pathway, and the overwhelmed proteolytic system eventually results in rod death. In the OS disc membranes, the photobleached rhodopsin finally releases all-*trans*-retinal and converts to rod opsin for pigment regeneration. The rod opsin mutants, lacking any ligand to stabilize its structure during pigment regeneration, is highly vulnerable to misfolding; this misfolded rod opsin can disrupt the OS membrane structure and add to rod stress.

Pharmacological strategies to rescue rods by restoring rhodopsin homeostasis: The pharmacological strategies discussed here are targeting on restoring rhodopsin homeostasis in preventing rod death for early and mid-stage adRP. Strategies aimed at preserving central vision and restoring vision in advanced RP are discussed in other reviews such as (Dalkara et al., 2016).

Chaperones stabilize the native folding of mutant rhodopsins: One strategy is to support the folding of the mutant rhodopsin by boosting the molecular chaperone activity, or by delivering small molecule chaperones (**Figure 1B**). While supplementation with vitamin A has shown efficacy in delaying disease progression in some RP patients, the lack of genetic diagnosis in these early studies makes the correlation of vitamin A with specific RP variants difficult. Genetic overexpression of molecular chaperones such as the 78-kDa glucose-regulated protein (GRP78/Bip) showed retinal protection in the transgenic rats expressing RHO^{P23H} (Gorbatyuk et al., 2010), thus supporting the hypothesis that photoreceptors can be rescued by providing sufficient support during folding. Based on this notion, chemical chaperones (4-phenylbutyric acid) and retinoid analogues (11-*cis*-locked retinals, retinobenzaldehydes, beta-ionone, and NSC45012) have been tested *in vitro* with the demonstration of folding improvement of the mutant rod opsin and increased transport to the plasma membrane in mammalian cells (Athanasiou et al., 2018). However, most of these small molecule chaperones have no published records of *in vivo* efficacy notwithstanding a recent report of daily oral 4-phenylbutyric acid being retinal-protective in the $Rho^{P23H/+}$ knock-in mouse model (Qiu et al., 2019).

To overcome this barrier, we used an unbiased cell-based high-throughput screening method to screen over 69,000 small molecules and identified a group of small molecules, including YC-001, as non-retinoid chaperones of rod opsin protein. We found YC-001 rescued the cellular transport of multiple misfolded rhodopsin mutants from ER to the plasma membrane with micromolar potency and low cytotoxicity. Unlike 9-*cis*-retinal, YC-001's chaperone activity is light-independent. The advantage of developing a non-retinoid chaperone is that it binds to rod opsin via non-covalent interactions to provide extra structural support for opsin while the native 11-*cis*-retinal is not sufficient. Additionally, it does not prohibit the visual pigment formation that is essential for phototransduction and visual function. We showed that a single intraperitoneal injection of YC-001 successfully protects the $Abca4^{-/-}$, $Rdh8^{-/-}$ double knockout mice from bright light damage, suggesting an *in vivo* retinal protective effect of YC-001 (Chen et al., 2018). Due to the short serum half-life of YC-001, we are actively working to optimize the chemical properties of YC-001 analogues to improve its stability *in vivo* for testing its chronic retinal protection in the $RHO^{P23H/+}$ knock-in mouse model of adRP.

Pharmacological clearance of misfolded rhodopsin: Alternative to rescuing folding, we also seek to rescue rods by inducing the degradation of the misfolded rhodopsin protein (**Figure 1B**). Previous studies showed that selectively digesting the mRNA of the mutant *RHO* by gene delivery of a hammerhead ribozyme exhibited long-term retinal protection in the RHO^{P23H} transgenic

rats (Lewin et al., 1998). Genetically inducing the proteasomal activity by overexpressing a regulatory subunit of the proteasome complex also showed strong retinal protection in the *RHO*$^{P23H/+}$ knock-in mouse model (Lobanova et al., 2018). Collectively, these studies suggest rods can be rescued by selectively reducing the rhodopsin mutant while the wild type (WT) RHO allele maintains retinal function. Based on this hypothesis, we undertook a comprehensive drug discovery campaign looking for small molecules that selectively induce the degradation of the misfolded rhodopsin to find an effective pharmacological treatment of *RHO*-associated adRP (Liu et al., 2020).

Using a luciferase reporter assay (**Figure 2A**), we performed a small-molecule high-throughput screening of 68,979 compounds (**Figure 2B**) and identified 9 compounds that selectively reduced the misfolded P23H rod opsin without an effect on the wild type rod opsin protein. Further, we found that five of these compounds, including methotrexate (MTX), promoted the degradation of misfolded rod opsin mutants. MTX, a drug commonly used off-label for treating retinal edema, already has rich documentation of its ocular pharmacokinetics and safety. MTX also showed higher selectivity in clearing mutant than WT rhodopsin (**Figure 2C**). Given this evidence, we selected MTX for a pilot study investigating its mechanism of action and efficacy. By pharmacologically blocking the proteasomal and the lysosomal pathways, we showed MTX induced degradation of P23H rod opsin via the lysosomal, but not the proteasomal pathway in the NIH3T3 cells. An intravitreal injection of MTX also increased the autophagy flux *in vivo* in the *Rho*$^{P23H/+}$ mouse retina, suggesting MTX clears misfolded rhodopsin mainly via the autophagy-lysosome pathway. Importantly, one intravitreal injection of 25 pmol MTX at postnatal day (PND15) increased electroretinogram (ERG) response and rhodopsin level in the retinae of *Rho*$^{P23H/+}$ knock-in mice at one month of age. Further, four weekly intravitreal injections increased the photoreceptor cell number in the retinae of *Rho*$^{P23H/+}$ mice compared to vehicle control. Our study showed a therapeutic potential of repurposing MTX for the treatment of RHO-associated RP.

MTX boosted the selective clearance of *RHO*^{P23H} *in vitro* but increased total rhodopsin level *in vivo* in the *Rho*$^{P23H/+}$ mouse retina. We showed that the autophagy flux was increased 48 hours after an intravitreal injection of MTX in the *Rho*$^{P23H/+}$ mice, thus these counter-intuitive observations are actually not conflicting. The increased rhodopsin immunofluorescence by MTX treatment was mainly on the OS layer, but not the outer nuclear layer, suggesting the rhodopsin was properly folded and transported and thus it was mainly the

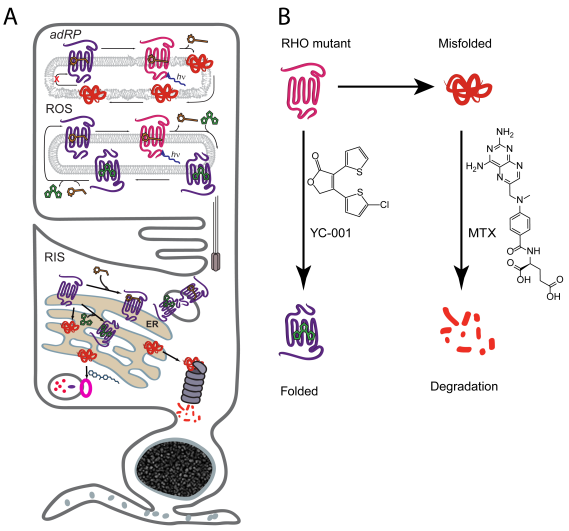

Figure 1 | Two pharmacological strategies to restore rhodopsin homeostasis for treating RHO-associated retinitis pigmentosa.
(A) Misfolded rhodopsin disrupts the homeostasis of rod cells by overwhelming the proteolytic system as well as disrupting the rod outer segment disc membrane. Pharmacological interventions include chaperones and inducers of lysosomal degradation are under development. (B) To restore rhodopsin protein homeostasis and prevent rod cell death, two pharmacological strategies were developed to either support the native folding of rhodopsin by a non-retinoid chaperone, such as YC-001, or to enhance the selective degradation of the misfolded rhodopsin mutant by small molecules such as MTX. adRP: Autosomal dominant retinitis pigmentosa; ER: endoplasmic reticulum; MTX: methotrexate; RHO: RHODOPSIN; RIS: rod inner segments; ROS: rod outer segments.

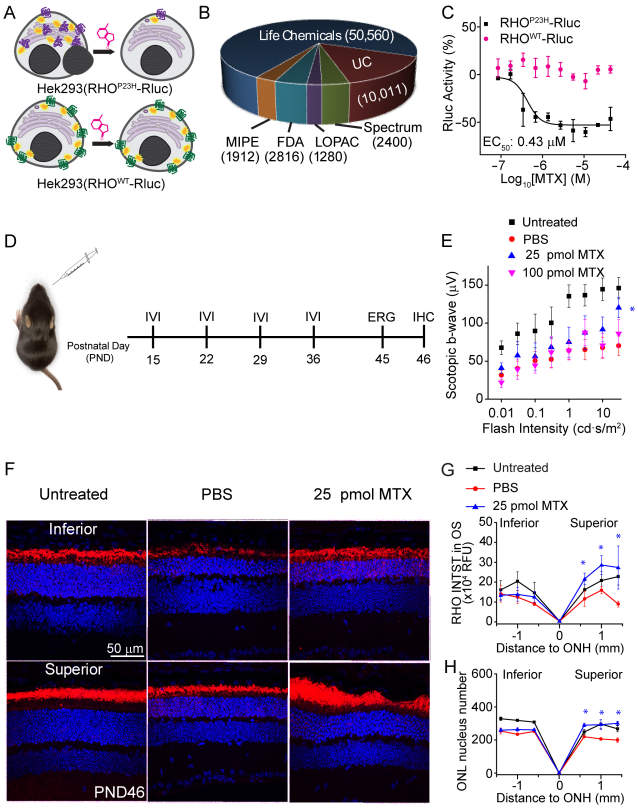

Figure 2 | Identification of methotrexate (MTX) as a selective enhancer of misfolded rhodopsin degradation which showed retinal protection in the mouse model of *RHODOPSIN* (*RHO*) associated autosomal dominant retinitis pigmentosa (adRP).
(A) A diagram of the high-throughput screen (HTS) assay using the luciferase reporter to screen for small molecules that selectively reduce the P23H but not the wild type (WT) rhodopsin expressed in the Hek293 stable cell lines. (B) Pie chart showing the composition of the 68,979 small molecules we screened from six compound collections including the 50k Life Chemicals, University of Cincinnati 10k compound collection (UC), the Spectrum collection, the Library of Pharmacologically Active Compounds (LOPAC), Food and Drug Administration approved drug collection (FDA), and the Mechanism Interrogation PlatE collection (MIPE). (C) The dose response curves of MTX in the luciferase reporter assay showing a selective activity towards the P23H rhodopsin clearance with an EC_{50} at 0.43 μM, but not towards WT rhodopsin level. Data and errors are means ± SD (n = 3). (D) The *Rho*$^{P23H/+}$ knock-in mice were administered with MTX by four weekly intravitreal injections (IVI) starting at postnatal day (PND) 15, and retinal function were recorded by electroretinography (ERG) at PND 45 and eyes were collected for immunohistochemistry (IHC). (E) *Rho*$^{P23H/+}$ Eyes treated with 25 pmol MTX showed increased scotopic ERG b-wave response compared to PBS-treated or 100 pmol MTX treated eye. Data and errors are means ± SEM (n = 6). (F) IHC images of *Rho*$^{P23H/+}$ mouse retinae untreated, or treated with phosphate-buffered saline (PBS) vehicle control, and 25 pmol of MTX. Rhodopsin (RHO), lectin peanut agglutinin (PNA) and nucleus (Hoechst 33342) were stained in red, green and blue, respectively. Scale bar: 50 μm. (G–H) Spider diagrams of RHO intensity in the outer segment (OS) layer and the outer nuclear layer (ONL) nucleus number per 200 μm length of retina cross section images taken at 0.6, 1 and 1.4 mm from optic nerve head (ONH). Data and errors are means ± SEM (n = 3). Reprinted with permission from Liu et al. (2020).

WT rhodopsin. Co-expressed with the misfolded rhodopsin mutant, WT rhodopsin co-aggregates in the ER in mammalian cell culture and can get degraded by the ERAD pathway. Thus, by selectively inducing the degradation of the misfolded rhodopsin mutant, more WT rhodopsin was transported to the OS layer by MTX *in vivo*. The direct molecular target of MTX that boosted rhodopsin degradation is still unknown and requires further investigation.

In addition to MTX, we also identified four other compounds that selectively increased misfolded rod opsin's degradation *in vitro*. These compounds include: CL-001 (Pubchem ID 11715767, EC_{50} = 2.1 μM by the *Rho*^{P23H} immunostaining and high-content imaging assay), CL-002 (Pubchem ID-6224422, EC_{50} = 1.6 μM), CL-005 (Pubchem ID10091681, EC_{50} = 1.3 μM) and CL-007 (Pubchem ID-5330790, EC_{50} = 2.9 μM). Whereas CL-002 has no record on pharmacological activities, CL-001 and CL-007 are both pan-inhibitors of cyclin-dependent kinases (CDKs), and CL-005 is an inhibitor of the prolyl hydroxylase, thus a stabilizer of heat shock-induced factor 1α (HIF1α). Even though cheminformatics of these hits indicate that CDKs may regulate rhodopsin degradation, reports about the retinal toxicity of CDK inhibitors (Illanes et al., 2006) suggest further identification of the specific CDK or other kinase that regulates rhodopsin degradation and development of selective CDK inhibitors in the future. Preconditioned hypoxia has shown protection against retinal degeneration in rodents (Grimm et al., 2005) and prolyl hydroxylase inhibitors have shown cellular protection from harmful conditions *in vitro* by inducing autophagy (Singh et al., 2020). Future investigations will test the hypothesis of autophagy-mediated rhodopsin degradation by stabilizing Hif1α and evaluate its potential for treating RP.

Immune response and retinal degeneration: Immune responses are associated with retinal degeneration. Microglia serve as innate immune surveillance and they play a neurotrophic role in phagocytosing dead cell bodies in retinal degeneration. Activated microglia produce a large amount of proinflammatory cytokines, recruiting myeloid cells, and their infiltration to the degenerative retina may contribute to photoreceptor loss (Rashid et al., 2019). MTX is a potent inhibitor of dihydrofolate reductase, this lends to its use in treating various cancers at high doses and rheumatoid arthritis at low dose (Maksimovic et al., 2020). At low dose, MTX shows strong anti-inflammatory effects by increasing adenosine release which suppresses activities of the immune cells including neutrophils, monocytes/macrophages, dendritic cells, and lymphocytes. Clinically, MTX is intravitreally injected to treat ocular inflammations such as retinal edema. We do not know yet whether the retinal protection by MTX in the *Rho*$^{P23H/+}$ mice *in vivo* is also due to the potent anti-inflammatory effects, and this question will be addressed soon.

Conclusion and perspective: Rhodopsin misfolding due to genetic mutation disrupts the protein homeostasis in rods and leads to adRP. We have developed two pharmacological strategies to restore the homeostasis of this highly abundant membrane protein. The non-retinoid chaperones, such as YC-001, provide additional structural support to the rod opsin mutants via non-covalent interactions, which rescue the folding and transport of the rod opsin mutants to the target membrane without blocking pigment regeneration during the visual cycle. Alternatively, degradation inducers, such as MTX, selectively clear the misfolded rhodopsin protein, providing improved homeostasis for WT rhodopsin and a longer lifespan of rods. Future studies will be focused on improving the chemical properties of these two groups of compounds to increase retinal bioavailability and half-life, or the development of slow ocular release formula to achieve long-term efficacy and safety for treating *RHO*-associated adRP.

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